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## Experimental studies on signal transduction pathways in rheumatoid arthritis

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**Differential influence of p38 mitogen-activated protein kinase (MAPK) inhibition on acute phase protein synthesis in human hepatoma cell lines and human liver slices**

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*Submitted*

## ABSTRACT

Inhibition of intracellular signal transduction is considered to be an interesting target for therapy in inflammation. Especially p38 MAPK inhibitors have been developed and are now in phase II clinical trials for rheumatoid arthritis. This study was designed to investigate the influence of p38 MAPK inhibition on acute phase protein (APP) production, which is dependent on both JAK/STAT and p38 MAPK pathways. We investigated the effects of p38 MAPK inhibition on APP production and mRNA expression in four human hepatoma cell lines, after stimulation with IL-6 and/or IL-1 $\beta$  or TNF- $\alpha$ . These effects were also investigated in human liver slices, a model to mimic the liver *in vivo*.

Two out of four cell lines produced C-reactive protein (CRP), especially after combined IL-6 and IL-1 $\beta$  stimulation. CRP production could be significantly inhibited by the p38 MAPK specific inhibitor RWJ 67657 at 1  $\mu$ M, which is pharmacologically relevant. Fibrinogen production was also inhibited at 1  $\mu$ M in all cell lines. Serum amyloid A (SAA) was produced in all four lines and was however not inhibited at 1  $\mu$ M. In liver slices increased production of APP was detected after stimulation, but p38 MAPK inhibition reduced only fibrinogen production. Concluding we found that production and mRNA expression of CRP and fibrinogen, but not SAA production and expression, significantly were inhibited by p38 MAPK specific inhibitor in hepatoma cell lines. We think that in the case of p38 MAPK inhibitor therapy in rheumatoid arthritis SAA might be a better marker for disease activity than CRP and fibrinogen, because SAA is not directly affected by p38 MAPK inhibition.

**Key words.** CRP, p38 MAPK, SAA, hepatoma cells

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which leads to the destruction of cartilage and bone in the joints. Cytokines like interleukin (IL)-1 $\beta$  and tumour-necrosis factor (TNF)- $\alpha$  are major players in the pathogenesis of RA<sup>1</sup>. During inflammation a number of physiological and metabolic changes, distant from the site of inflammation, are induced which collectively are called the acute phase response. This response is characterized by the change in concentration of many plasma proteins<sup>2</sup>. The liver is a major target of systemic inflammatory mediators and is responsible for supplying many components for defence at the site of tissue damage, and is the producer of the acute phase plasma proteins (APP) after stimulation by cytokines<sup>3</sup>. Measurement of the APP serum CRP (C-reactive protein) is useful in managing disease, since the concentration reflects the inflammatory status of a patient. In RA, serial measurements of CRP are of prognostic value<sup>4</sup>. The most familiar indicator of the response of the acute phase proteins is the erythrocyte sedimentation rate (ESR), which largely depends on the concentration of fibrinogen.

IL-6 is recognized as the principal regulator of most APP genes (the so-called type-2 or IL-6-specific APPs), including the three chains of fibrinogen, haptoglobin, and the protease inhibitors  $\alpha$ 1-antichymotrypsin,  $\alpha$ 1-antitrypsin and  $\alpha$ 2-macroglobulin. The IL-6 like cytokines IL-11, oncostatin M (OSM), leukaemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) induce type-2 APPs in a similar way. Type-1 APPs are regulated by the IL-1-like cytokines and include  $\alpha$ 1-acid glycoprotein (AGP), complement C3, serum amyloid A (SAA) and CRP. TNF- and IL-1-mediated stimulation of type-1 genes is synergistically enhanced by IL-6-like cytokines, while the production of IL-6-dependent (type-2) APPs usually is inhibited by IL-1-like cytokines<sup>3</sup>. The IL-6-like cytokines bind to plasma membrane receptor complexes containing the common signal transducing receptor chain gp 130 (glycoprotein 130). Signal transduction involves the activation of JAK (Janus kinase) tyrosine kinase family members, leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family<sup>5</sup>. Upon activation, STAT proteins dimerize, translocate to the nucleus, and initiate transcription of the STAT-responsive genes. The importance of the JAK/STAT pathway in RA has not completely been established yet, but STAT1 and STAT3 seem to have both protective and pathogenic properties<sup>6</sup>, with a regulating role for the suppressors of cytokine signalling (SOCS)<sup>7</sup>. Dimerization of IL-6-type cytokine receptor not only activates the JAK/STAT pathway, but may also stimulate the mitogen-activated protein kinase (MAPK) cascade through activation of Ras, a GTP-binding protein<sup>5</sup>. Simultaneous activation of the JAK/STAT and MAPK pathways in a rat hepatoma cell line has also been described for IL-22, an IL-10-related cytokine<sup>8</sup>. A role for one of the MAPK homologues, p38 MAPK in IL-6 induced functions has been established in different studies<sup>9;10</sup>. Furthermore p38 MAPK is involved in the apoptotic pathway in human hepatoma cell lines, because inhibition of p38 MAPK leads to reduced apoptosis in hepatocellular carcinoma<sup>11</sup>. Activation of p38 MAPK in human hepatoma cells by TNF- $\alpha$ , leading to the production of RANTES, is correlated to alcoholic liver disease<sup>12</sup>.

p38 MAPK plays a dominant role in signal transduction pathways in inflammatory diseases and in the last years several specific inhibitors have been developed (for

review see <sup>13</sup>). The p38 MAPK inhibitor RWJ 67657 has been shown to significantly inhibit the release of TNF- $\alpha$  from lipopolysaccharide-treated human peripheral blood mononuclear cells <sup>14</sup>, but also from macrophages <sup>15</sup>. This compound is approximately 10-fold more potent than the reference standard p38 MAPK inhibitor SB 203580 in all p38 MAPK dependent in vitro systems tested. RWJ 67657 specifically inhibits the enzymatic activity of recombinant p38 $\alpha$  and  $\beta$ , but not of  $\gamma$  and  $\delta$  in vitro, and has no significant activity against a variety of other kinases <sup>14</sup>. Since p38 MAPK inhibitors are now in phase II clinical trial for RA <sup>16</sup>, it is important to know whether these inhibitors have a direct effect on the production of acute phase proteins, due to cross talk between the JAK/STAT and p38 MAPK cascades, in order to elucidate whether these APP are still a valuable marker for the disease activity during treatment with p38 MAPK inhibitors.

In this study, we investigated the effect of p38 MAPK inhibition on IL-6, IL-1 $\beta$  and TNF- $\alpha$  induced acute phase protein production in four different hepatoma cell lines both at the level of mRNA expression and at the level of protein production. The effects on CRP, SAA, complement factor 3 (C3), fibrinogen, and albumin were studied. For mRNA analysis we studied the SAA-1 gene. Fibrinogen was studied by analyzing fibrinogen- $\beta$  and fibrinogen- $\gamma$  genes. Fibrinogen- $\beta$  chain synthesis is considered to be the rate-limiting chain for assembly and secretion of mature fibrinogen. The promoter regions of both  $\beta$ - and  $\gamma$ -genes share the IL-6 responsive element, but the  $\gamma$ -gene lacks the C/EBP response element <sup>17</sup>.

To study the effects in a system that mimics the liver *in vivo*, we used human liver slices. The cellular architecture of the liver is retained in the human liver slices, making it the ideal in vitro preparation for multi-cellular processes. This model has been developed and validated for drug metabolism and toxicity studies <sup>18</sup>. In addition, this system was used to detect the organ source of the acute phase protein procalcitonin, and it was shown that procalcitonin, in addition to SAA and CRP, in man originates from the liver <sup>19</sup>.

## MATERIALS AND METHODS

### *Reagents*

RWJ 67657 was provided by Johnson and Johnson (R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ). The human hepatocellular carcinoma cell lines HepG2 and Hep3B, and the hepatoma cell line PLC/PRF/5 were purchased from the ATCC (American Type Culture Collection, Manassas, VA). The hepatoma cell line HuH7 was a kind gift from Dr. R. Kleemann (TNO, Leiden, The Netherlands). Recombinant human IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were from R&D Systems (Minneapolis, MN). Foetal calf serum (FCS) and DMEM (Dulbecco's-modified Eagle Medium) were obtained from Biowhittaker (Verviers, Belgium).

Specific antibodies to p38 MAPK, phospho-p38 MAPK, STAT3 and phospho-STAT3 were purchased from Cell Signalling Technologies (Beverly, MA) and detecting antibody peroxidase-swine-anti-rabbit IgG was from DAKO (Glostrup, Denmark). Antibodies for CRP and fibrinogen ELISA were obtained from DAKO, capture-antibodies for C3 ELISA were from Calbiochem (San Diego, CA) and detecting antibodies were from ICN Biomedicals (Irvine, CA). Antibodies and ELISA for SAA were developed in our laboratory <sup>20</sup>.

All reagents for RNA isolation and reverse transcriptase reaction were purchased from Invitrogen, Life Technologies (Gaithersburg, MD). Reagents for real-time RT-PCR were obtained from Applied Biosystems (Foster City, CA).

### ***Culture of hepatoma cell lines***

Cell lines were maintained in DMEM supplemented with 10% FCS and gentamycin in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. Hep3B and PLC/PRF/5 were passaged twice a week in a 1:3 ratio, HuH7 in a 1:10 ratio. HepG2 was passaged weekly in a 1:3 ratio. For experiments, the hepatoma cells lines were grown to confluence in 12 wells (1 ml) or 24 wells (0.5 ml) tissue culture plates (Corning, Schiphol, The Netherlands). Activation of the cells with 50 ng/ml IL-6 and/or 10 ng/ml IL-1 $\beta$  or 10 ng/ml TNF- $\alpha$  was performed in DMEM with 1% FCS and 0.1  $\mu$ M dexamethasone.

### ***Activation of p38 MAPK and STAT3***

Phosphorylation of p38 MAPK and STAT3 in hepatoma cell lines was analyzed by western blotting. Confluent cells were stimulated with IL-6, IL-1 $\beta$  or TNF- $\alpha$  for various periods of time (15 and 30 min, 1, 2, 4, 6, and 24 hours) in DMEM with 1% FCS and 0.1  $\mu$ M dexamethason. Cell extracts were prepared by lysing the cells with 200  $\mu$ l 1x SDS sample buffer (containing 2% SDS, 10% glycerol, 50 mM dithiothreitol, 62.5 mM Tris-HCl (pH 6.8) and 0.01% brome-phenol blue). Cells were scraped off the wells and the lysates were subsequently sonicated for 5-10 seconds and boiled for 5 minutes. The samples were loaded onto a 10% SDS-PAGE gel and run at 200 V and 15 Watt maximum. Semidry blotting was performed onto nitrocellulose membrane and immunodetection with anti-phospho-p38 MAPK was performed, followed by incubation with peroxidase-anti-rabbit IgG. Enhanced chemiluminescence (ECL) detection was performed according to the manufacturers guidelines (Lumi-Light<sup>plus</sup>, Roche Diagnostics, Mannheim, Germany). Blots were exposed to Hyperfilm<sup>TM</sup> (Amersham Biosciences, Roosendaal, The Netherlands) and developed. Subsequently, blots were stripped with Restore<sup>TM</sup> Western Blot Stripping Buffer (Pierce, Rockford, IL) and immunodetection with anti-p38 MAPK was performed. The same procedure was followed for anti-phospho-STAT3 and anti-STAT3 immunodetection.

### ***Production of acute phase proteins by hepatoma cell lines***

Cells were stimulated with IL-6, IL-1 $\beta$  and TNF- $\alpha$  alone or in combination during 48 hours. The effect of p38 MAPK inhibition was investigated by pre-incubation for 1 hour with a concentration range of RWJ 67657 (0, 0.01, 0.1, 1, and 10  $\mu$ M, diluted from stock solution of 10 mM in DMSO (dimethylsulfoxide)). Production of acute phase proteins was determined in the culture supernatants by ELISAs. For CRP and fibrinogen ELISA rabbit-anti-CRP (1:10000) or rabbit-anti-fibrinogen (1:4000) were coated in 96-well plates. Diluted supernatants were added with standards in a concentration range. After incubation conjugated rabbit-anti-CRP (1:2000) or -fibrinogen (1:4000) was added and detection was performed with the enzyme substrate tetramethyl-benzidin (TMB, Roth, Karlsruhe, Germany) and the reaction was stopped with 1M H<sub>2</sub>SO<sub>4</sub>. Plates were read in an ELISA reader at 450 nm and concentration of protein was determined with the SOFTmax PRO software (Molecular

Devices, Sunnyvale, CA). The detection limits for the assays are 0.1 ng/ml and 40 ng/ml for CRP and fibrinogen respectively. For complement C3 ELISA, goat-anti-human C3 (1:2000) and peroxidase-conjugated goat-anti-human-C3 (1:2000) was used. Detection and calculations were done as described for the CRP and fibrinogen ELISAs. The detection limit for the assay is 80 ng/ ml.

The SAA ELISA was performed as described before (15). Shortly, a capture monoclonal antibody (Reu.86.5, which reacts with all subtypes of SAA) was coated (1:1000), followed by incubation with diluted supernatants or standards. Detection was done with a peroxidase-conjugated monoclonal antibody (Reu.86.1, specific for SAA-1), followed by the substrate reaction. The detection limit of the assay is 2 ng/ ml.

### ***mRNA analysis of acute phase proteins***

Hepatoma cells (PLC/PRF/5 and Hep3B) were grown to confluence in 12 wells plates and stimulated for 24 hours as described above. One hour pre-treatment with 0, 0.1, 1, and 10  $\mu$ M RWJ 67657 was done for all stimulations. Total RNA was isolated from the cells with TRIzol reagent according to the manufacturers instructions (Invitrogen, Life Technologies). DNase treatment (Ambion, Huntingdon, Cambridgeshire, UK) was performed and subsequently cDNA was synthesized from 2.0  $\mu$ g of total RNA using M-MLV Reverse Transcriptase and oligo (dT)<sub>14-18</sub> (Invitrogen, Life Technologies). For the measurement of mRNA for albumin, CRP, SAA, C3, fibrinogen- $\beta$ , fibrinogen- $\gamma$  and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1  $\mu$ l of cDNA in duplicate was used for amplification by the Taqman real-time PCR system (ABI Prism 7900HT Sequence Detection System, Applied Biosystems) with specific Taqman primers/probes. The Assay-on-Demand numbers for the genes were as follows: albumin: Hs00609411\_m1, CRP: Hs00357041\_m1, C3: Hs00163811\_m1, fibrinogen- $\beta$ : Hs00170586, fibrinogen- $\gamma$ : Hs00241037\_m1, and GAPDH: Hs99999905\_m1. For SAA-1, suitable primers and probe were developed between the transit of exon 3 and 4 using the software program Primer Express 2.0 (Applied Biosystems) and the assay was ordered by Assay-by-Design.

Amplification was performed using standard conditions: denaturation at 95°C for 15 seconds, 40 cycles of amplification with annealing at 60°C for 1 minute, and extension at 50°C for 2 minutes. According to the comparative Ct (threshold cycle value) method described in the ABI manual, the resulting mRNA amount of the gene of interest was normalized to the housekeeping gene GAPDH, yielding the  $\Delta$ Ct value. The  $\Delta$ Ct value of unstimulated cells was subtracted from the average  $\Delta$ Ct value of each sample, yielding the  $\Delta\Delta$ Ct. The amount of target, normalized to an endogenous reference (GAPDH) and relative to the control sample, is given by:  $2^{-\Delta\Delta\text{Ct}}$ .

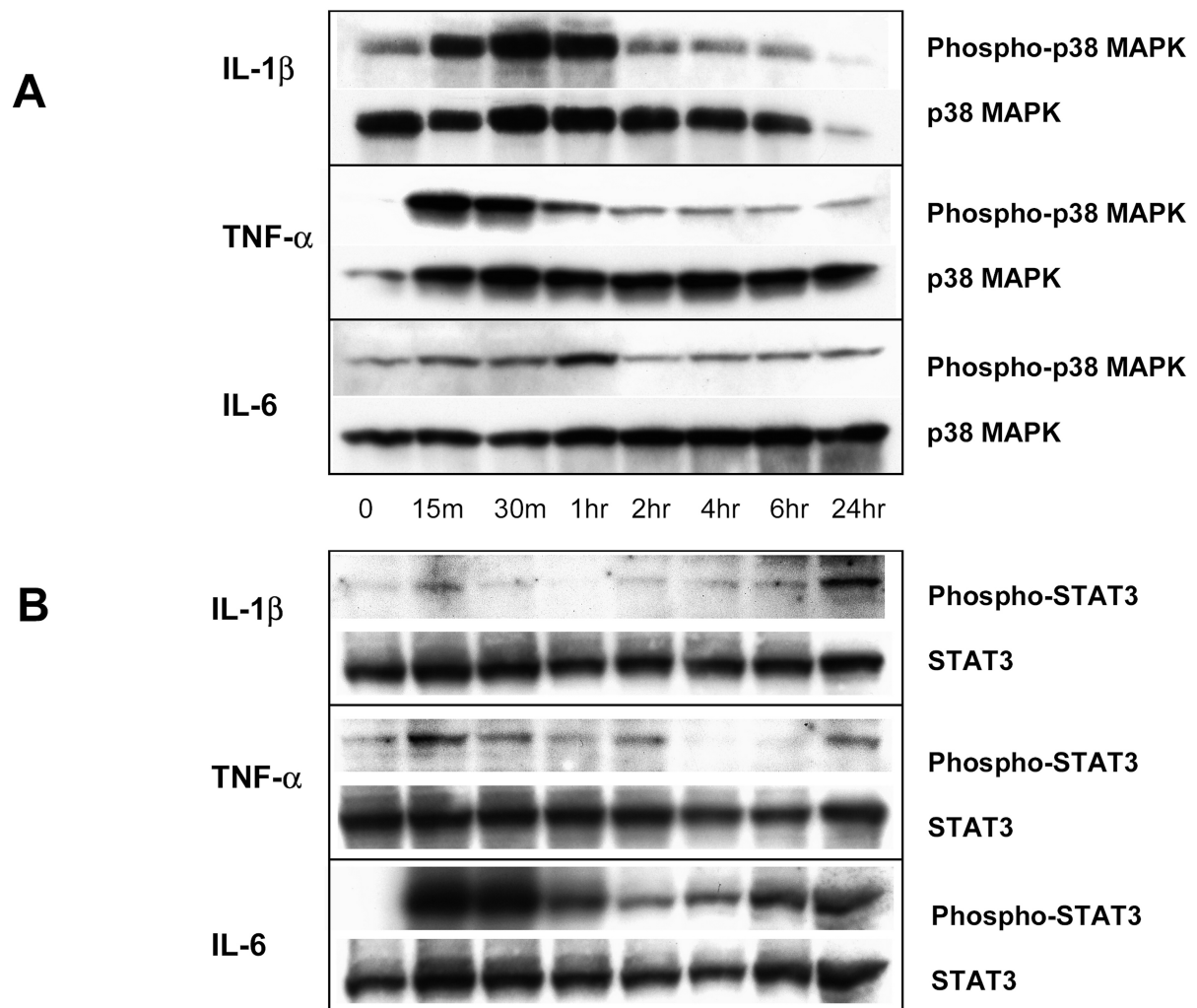
### ***Liver slices to mimic the liver in vivo***

Human liver tissue was obtained from livers procured from multi-organ donors. Consent from the legal authorities and from the families concerned was obtained from the explantation of organs for transplantation purposes. The human livers were handled as described before <sup>21</sup>. Liver tissue cores (diameter 8 mm) were prepared, and stored in ice-cold University of Wisconsin organ preservation solution until slicing. Liver slices (200-300  $\mu$ m thickness, wet weight 10-14 mg) were prepared with the Krumdieck slicer. The slices were incubated 24 hours in 3.2 ml Williams' medium E

supplemented with 25 mM glucose, 50 µg/ml gentamycin and 0.1 µM dexamethasone. The 6-well tissue culture plates were continuously rocked back and forth (90/min) at 37°C under 5% CO<sub>2</sub>/95% O<sub>2</sub>. Liver slices were stimulated in triplicate with 50 ng/ml IL-6 or 10 ng/ml IL-1β during 24 hours with or without pre-treatment for 1 hour with 1 µM RWJ 67657. Supernatants were collected for measurement of acute phase proteins. The triplicate slices were collected and homogenized in TRIzol for isolation of mRNA according to manufacturers instructions. mRNA expression of acute phase proteins was performed as described above for the hepatoma cell lines.

## STATISTICS

One-way ANOVA with Dunn's Multiple Comparison Test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA).



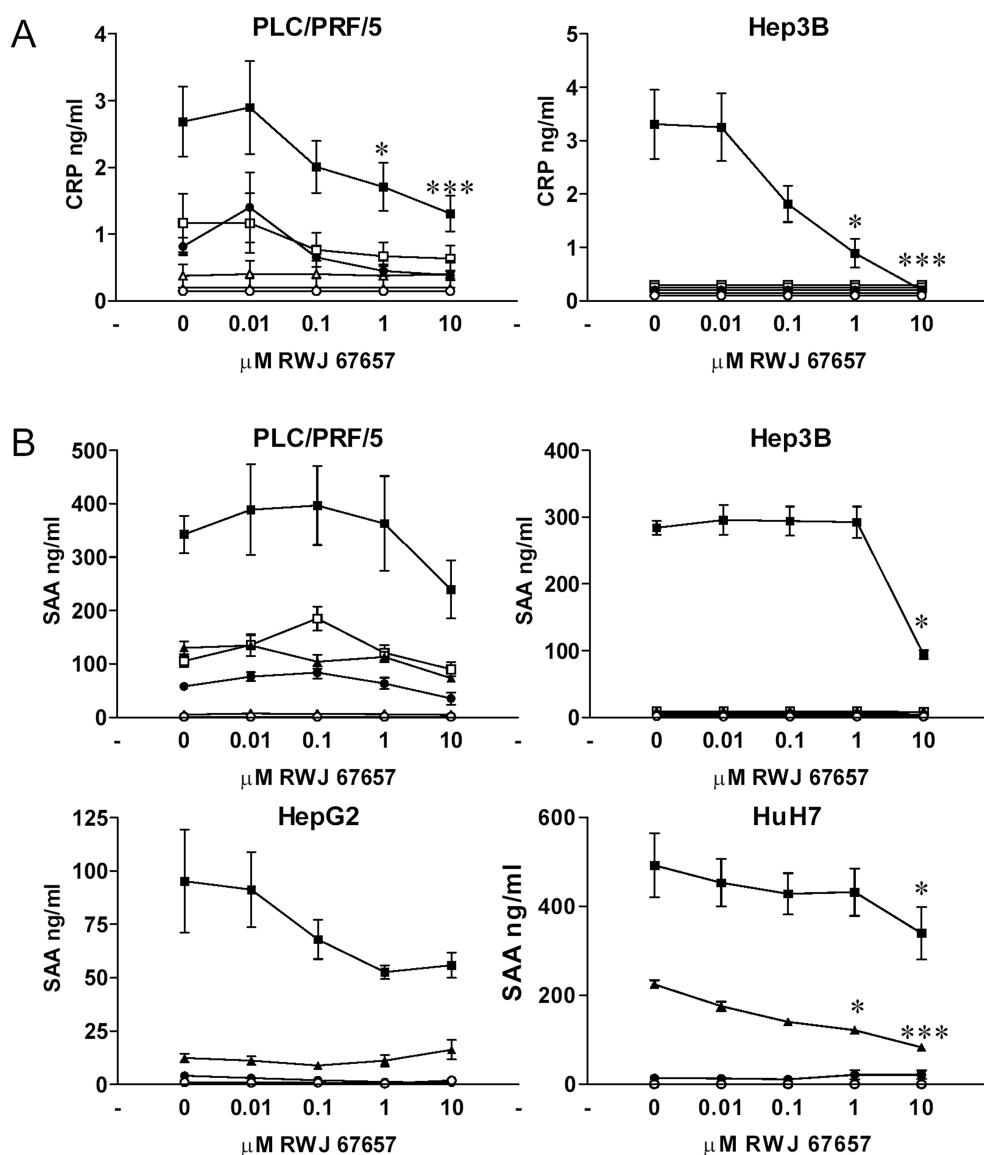
**Figure 1.** Phosphorylation of p38 MAPK (A) and STAT3 (B) in hepatoma cell line PLC/PRF/5 after stimulation with cytokines.

Hepatoma cells were stimulated with 10 ng/ml IL-1β or TNF-α or 50 ng/ml IL-6 in DMEM with 1% FCS and 0.1µM dexamethason for increasing periods of time. Phosphorylation was measured by Western blot using specific antibodies against p38 MAPK, phospho-p38 MAPK, STAT3 and phospho-STAT3.



## RESULTS

Activation of p38 MAPK and STAT3 phosphorylation of p38 MAPK and STAT3 in the cell lines Hep3B and PLC/PRF/5 was analysed after stimulation with IL-6, IL-1 $\beta$  and TNF- $\alpha$  after various periods of time. In figure 1 representative examples are shown of the Western blots. In figure 1A, a rapid phosphorylation of p38 MAPK is demonstrated after stimulation with IL-1 $\beta$  and TNF- $\alpha$ , but not after stimulation with IL-6. In figure 1B, STAT3 is strongly phosphorylated after IL-6 stimulation, whereas after IL-1 $\beta$  and TNF- $\alpha$  stimulation hardly any phosphorylation of STAT3 is detected.



**Figure 2.** Effect of RWJ 67657 pre-treatment on CRP (A) and SAA (B) production in hepatoma cell lines after stimulation with (combinations of) cytokines.

Cells were unstimulated ( $\circ$ ) or stimulated with IL-1 $\beta$  ( $\blacktriangle$ ), IL-6 ( $\bullet$ ), TNF- $\alpha$  ( $\Delta$ ), IL-1 $\beta$  + IL-6 ( $\blacksquare$ ) or TNF- $\alpha$  + IL-6 ( $\square$ ) for 48 hours, with or without pre-treatment of 0.01, 0.1, 1, and 10  $\mu$ M RWJ 67657. CRP and SAA levels were measured in cell supernatants by ELISA and expressed in ng/ml. In the graphs the mean and SEM is shown. (\* $p$  < 0.05, \*\*\* $p$  < 0.001, One-way ANOVA with Dunn's Multiple Comparison Test, tested against the stimulated control).

**Production of acute phase proteins by hepatoma cell lines**

In the supernatants of the 4 different hepatoma cell lines the production of the acute phase proteins CRP, SAA, fibrinogen and C3 was measured by ELISA. CRP (type-1 APP) production could not be detected in HepG2 and HuH7 cell lines. In PLC/PRF/5, highest CRP production was seen after combined IL-1 $\beta$  and IL-6 stimulation, and to a lesser extent after TNF- $\alpha$  stimulation alone or together with IL-6. In Hep3B, CRP production was found exclusively after simultaneous stimulation with IL-1 $\beta$  and IL-6 (figure 2A).

In both cell lines, CRP production was inhibited by RWJ 67657 both at 1 and 10  $\mu$ M. High production of SAA was detected in all hepatoma cell lines after combined stimulation with IL-1 $\beta$  and IL-6 (figure 2B). Inhibition due to p38 MAPK inhibition was seen only at 10  $\mu$ M in Hep3B and HuH7. No significant inhibition was seen in PLC/PRF/5 and HepG2.

**Table 1.** Effect of RWJ 67657 pre-treatment on fibrinogen production ( $\mu$ g/ml) in 4 hepatoma cell lines (mean of 6 experiments).

Cells were unstimulated or stimulated with (combinations of) cytokines for 48 hours, with or without pre-treatment of 0.01, 0.1, 1, and 10  $\mu$ M RWJ 67657. Fibrinogen levels were measured in cell supernatants by ELISA and expressed in  $\mu$ g/ml. The table shows the mean and SEM (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, One-way ANOVA with Dunn's Multiple Comparison Test, tested against the stimulated control). (BD = below detection limit, ND = not determined).

	unstim	IL-1 $\beta$	IL-6	IL-1 $\beta$ + IL-6	TNF- $\alpha$	IL-1 $\beta$ + TNF- $\alpha$
<b><math>\mu</math>M RWJ</b>	<b>PLC/PRF</b>					
0	2.77	2.13	10.33	5.92	2.35	8.77
0.01	2.47	1.77	10.42	5.57	2.05	8.00
0.1	2.68	1.65	8.67	5.12	1.87	7.20
1	2.35	1.45	8.23	4.57	1.48	5.58*
10	1.68	0.85	5.10*	2.48**	0.95	2.57***
<b><math>\mu</math>M RWJ</b>	<b>Hep3B</b>					
0	BD	BD	1.62	0.58	BD	0.35
0.01			1.25	0.62		0.30
0.1			0.80	0.43		0.20
1			0.60*	0.28		0.13
10			0.27***	0.10**		0.10
<b><math>\mu</math>M RWJ</b>	<b>HepG2</b>					
0	5.21	5.09	20.38	20.30	ND	ND
0.01	4.82	5.45	18.08	17.61		
0.1	4.30	5.40	17.97	16.58		
1	3.93	4.41	15.15	11.44*		
10	2.65	2.70	9.71*	5.73***		
<b><math>\mu</math>M RWJ</b>	<b>HuH7</b>					
0	2.88	2.90	10.93	5.77	ND	ND
0.01	2.23	2.53	9.82	5.56		
0.1	2.25	2.27	8.12	6.00		
1	2.08	1.81	7.70*	4.47*		
10	1.78	1.25	6.30***	2.56**		

Results of fibrinogen production are shown in table 1. Fibrinogen is constitutively produced in the liver and in cultured hepatoma cell lines as well. Fibrinogen is a type-2 APP, and after stimulation with IL-6 a maximal fourfold induction of production is seen. In Hep3B cells fibrinogen production was not constitutive and could only be detected after IL-6- or combined stimulation. Significant inhibitory effects of p38 MAPK at 10  $\mu$ M were seen in all 4 cell lines after IL-6 stimulation alone or in combination of IL-6 and IL-1 $\beta$ , and in Hep3B, HepG2 and HuH7 at 1  $\mu$ M, but not in PLC/PRF/5.

In table 2, we show the results of C3 production in the hepatoma cell lines. Like fibrinogen, C3 is also constitutively produced, and production is induced after stimulation with all cytokines and combinations. Significant inhibition at 10  $\mu$ M RWJ 67657 was seen in all 4 cell lines after IL-1 $\beta$  stimulation and in Hep3B and HepG2 after IL-1 $\beta$  + IL-6 stimulation. In HepG2, there was also significant inhibition of IL-6 induced C3 production at 1  $\mu$ M. Pre-treatment of hepatoma cells with 0.1% DMSO had no significant effect on any acute phase protein production (data not shown).

**Table 2.** Effect of RWJ 67657 pre-treatment on C3 production ( $\mu$ g/ml) in 4 hepatoma cell lines (mean of 6 experiments).

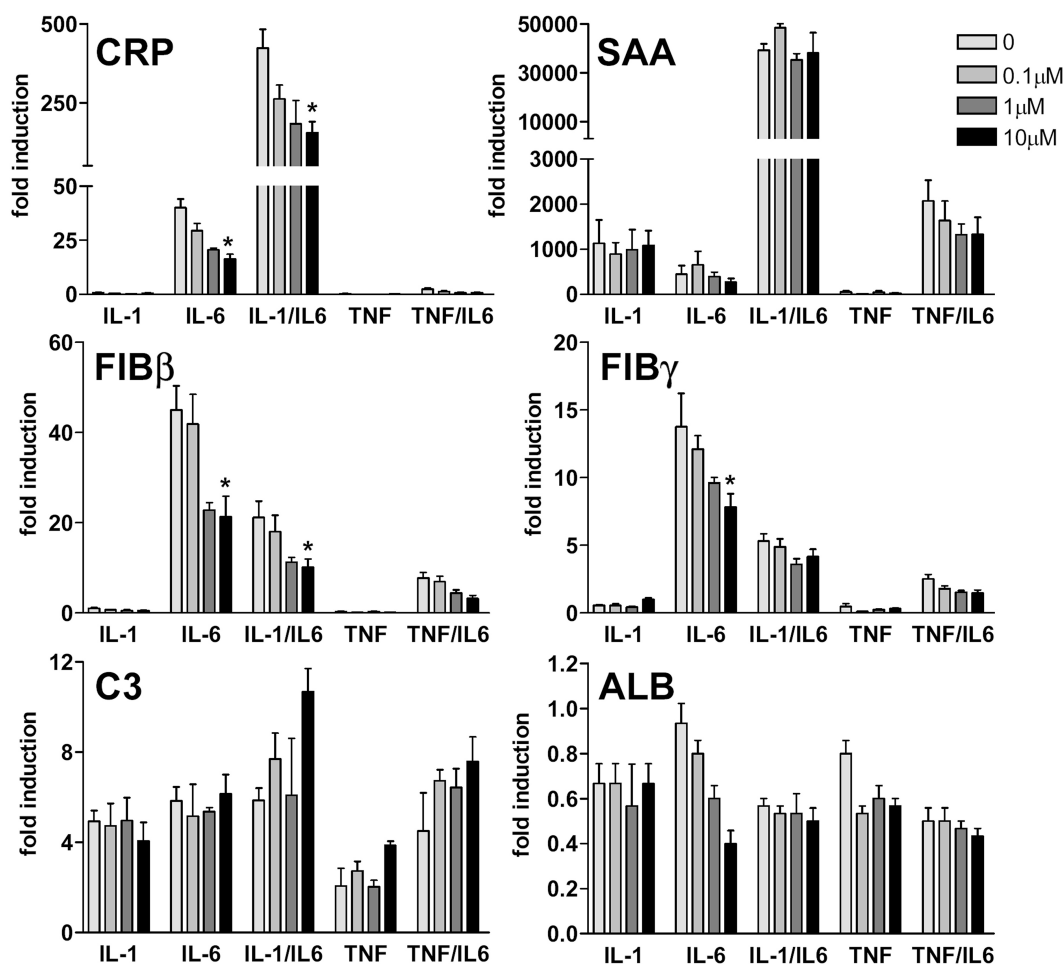
Cells were unstimulated or stimulated with (combinations of) cytokines for 48 hours, with or without pre-treatment of 0.01, 0.1, 1, and 10  $\mu$ M RWJ 67657. C3 levels were measured in cell supernatants by ELISA and expressed in  $\mu$ g/ml. The table shows mean and SEM(\*  $p < 0.05$ , \*\*  $p < 0.01$ , One-way ANOVA with Dunn's Multiple Comparison Test, tested against the stimulated control). ND = not determined.

	unstim	IL-1 $\beta$	IL-6	IL-1 $\beta$ + IL-6	TNF- $\alpha$	IL-1 $\beta$ + TNF- $\alpha$
<b><math>\mu</math>M RWJ</b>	<b>PLC/PRF</b>					
0	0.72	1.57	2.03	1.62	1.22	1.47
0.01	0.73	1.28	1.37	1.37	1.25	1.52
0.1	0.65	1.20	1.65	1.33	1.12	1.45
1	0.60	1.07*	1.45	1.55	0.98	1.25
10	0.52	0.82**	1.55	1.12	0.72	0.88
<b><math>\mu</math>M RWJ</b>	<b>Hep3B</b>					
0	0.82	3.13	3.85	5.27	1.80	3.23
0.01	0.67	3.72	5.27	6.00	2.05	3.17
0.1	0.65	3.42	4.37	6.00	1.82	2.95
1	0.65	2.57	2.67	4.97	1.48	2.48
10	0.55	1.72**	2.45	2.28*	1.32	1.95
<b><math>\mu</math>M RWJ</b>	<b>HepG2</b>					
0	5.58	11.12	7.33	9.25	ND	ND
0.01	4.70	12.95	6.77	7.63		
0.1	5.22	11.53	8.30	7.45		
1	4.70	8.70	5.77*	6.88		
10	3.83	6.40*	4.97**	4.57*		
<b><math>\mu</math>M RWJ</b>	<b>HuH7</b>					
0	0.48	2.67	1.42	2.83	ND	ND
0.01	0.47	3.02	1.30	2.28		
0.1	0.58	2.87	1.42	2.53		
1	0.75	2.47	1.77	2.22		
10	1.23	1.52*	0.92	2.07		

### mRNA analysis of acute phase proteins

With quantitative RT-PCR mRNA expression of CRP, SAA-1, fibrinogen- $\beta$ , fibrinogen- $\gamma$ , complement C3, and albumin was measured in Hep3B and PLC/PRF/5. As previously mentioned CRP production by HepG2 and HuH7 cells could not be detected. In addition, CRP mRNA expression was not found in these 2 cell lines, and therefore the experiments were performed with the 2 other cell lines. Figure 3 shows the results of mRNA expression of 3 different experiments in Hep3B cells after 24-hour stimulation.

Results of mRNA analysis in PLC/PRF/5 were comparable. CRP and SAA-1 mRNA expressions were abundantly induced, especially after combined stimulation with IL-6 and IL-1 $\beta$ . Pre-treatment with 0.1, 1 or 10  $\mu$ M RWJ 67657 reduced CRP mRNA expression in a dose dependent way, but not SAA-1 mRNA expression. Reduction of



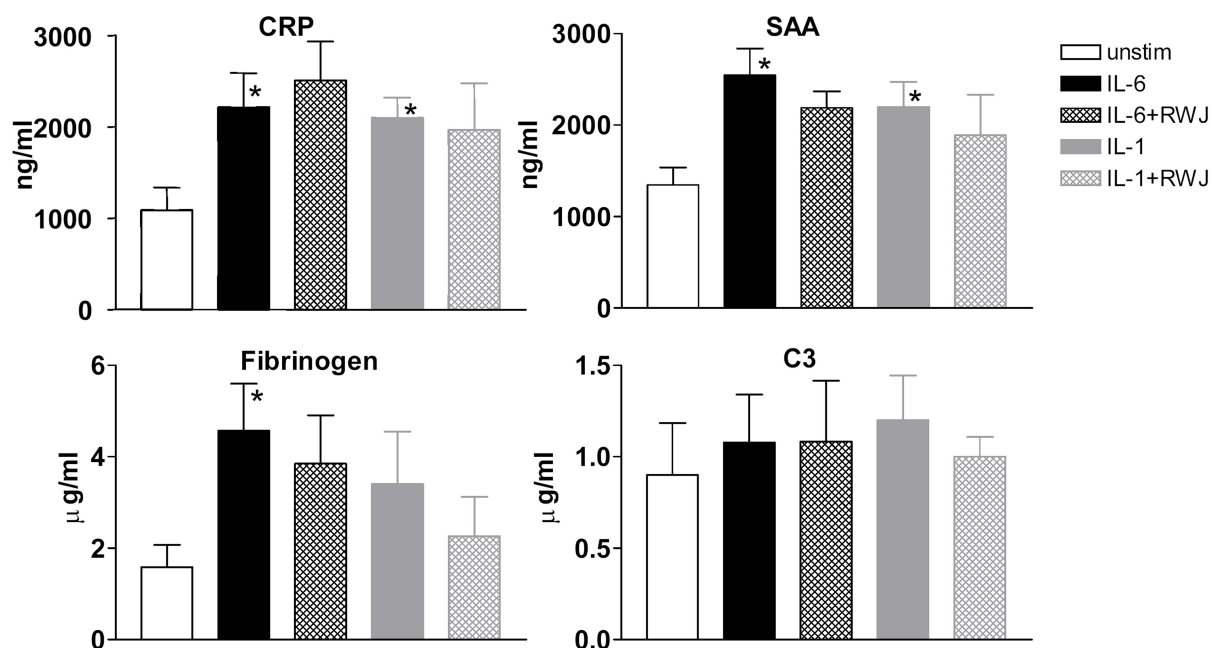
**Figure 3.** Effect of RWJ 67657 pre-treatment on mRNA expression of CRP, SAA-1, fibrinogen- $\beta$ , fibrinogen- $\gamma$ , complement C3 and albumin in Hep3B cells after stimulation with (combinations of) cytokines.

Cells were stimulated with IL-1 $\beta$ , IL-6, IL-1 $\beta$  + IL-6, TNF- $\alpha$ , and TNF- $\alpha$  + IL-6 for 24 hours with or without pre-treatment with 0.1, 1, and 10  $\mu$ M RWJ 67657. mRNA expression was determined with real-time RT-PCR, results are expressed as fold induction compared to unstimulated cells (fold induction = 1). Bars show mean ( $n=3$ ) and SEM. (\* $p<0.05$ , One-way ANOVA with Dunn's Multiple Comparison Test, tested against the stimulated control).

CRP mRNA at 10  $\mu$ M was significant both after IL-6 and combined IL-6/IL-1 $\beta$  stimulation. Fibrinogen and C3 mRNA are constitutively expressed, but can be induced by cytokine stimulation. Fold inductions after IL-6, combined IL-6/IL-1 $\beta$  and combined IL-6/TNF- $\alpha$  stimulation for fibrinogen- $\beta$  and fibrinogen- $\gamma$  were 45.0, 21.0, 7.7 and 13.8, 5.3, 2.5 respectively. mRNA induction for both fibrinogen-chains was significantly reduced by 10  $\mu$ M p38 MAPK treatment after IL-6 stimulation. C3 mRNA expression was induced after all stimulations, but there was no inhibitory effect detectable due to pre-treatment with RWJ 67657. Albumin mRNA expression was reduced in all stimulated cells, and was further reduced with 10  $\mu$ M p38 MAPK inhibitor in IL-6-stimulated cells.

***Effect of RWJ 67657 on protein production and mRNA expression of acute phase proteins in human liver slices***

The levels of the acute phase proteins CRP, SAA, fibrinogen and C3 were determined in the supernatants of liver slices which were stimulated with IL-6 or IL-1 $\beta$ , with or without pre-treatment with 1  $\mu$ M RWJ 67657. In figure 4 the results are shown of 2 to 4 experiments, performed in triplicate. In unstimulated cultures, very high levels of CRP and SAA were present: 1090  $\pm$  247 ng/ml for CRP and 1346  $\pm$  193 ng/ml for SAA.



**Figure 4.** Acute phase protein production in human liver slices.

CRP, SAA, fibrinogen and C3 levels were determined by ELISA in liver slice supernatants, after 24 hours stimulation with 50 ng/ml IL-6 or 10 ng/ml IL-1 $\beta$  with or without pre-treatment with 1  $\mu$ M RWJ 67657. Experiments ( $n=2-4$ ) were performed in triplicate, bars show mean and SEM. (\* $p<0.05$ , One-way ANOVA with Dunn's Multiple Comparison Test, tested against the unstimulated control).

Stimulation raised the CRP and SAA levels to 2212  $\pm$  376 ng/ml and 2546  $\pm$  193 ng/ml after IL-6 stimulation, and 2101  $\pm$  220 ng/ml and 2199  $\pm$  274 ng/ml after IL-1 $\beta$  stimulation.

The induced levels differ significant ( $p < 0.05$ ) from the levels in unstimulated slices. Pre-treatment with 1  $\mu\text{M}$  RWJ 67657 had no effect on the CRP and SAA levels. Fibrinogen was induced more than two-fold after stimulation (significant after IL-6 stimulation) and there was an inhibitory effect of p38 MAPK treatment, although not significant. Complement C3 levels were not induced and not inhibited in the liver slices.

The expression of CRP, SAA-1, fibrinogen- $\beta$  and - $\gamma$ , and C3 mRNA in liver slices was conform the protein expression (data not shown). Unstimulated samples already had a high CRP and SAA-1 mRNA expression, which could be seen from the low number of cycli relative to GAPDH needed in the RT-PCR and expression was less induced by stimulation compared to the hepatoma cell lines. Albumin mRNA expression was reduced after cytokine treatment. No effects of p38 MAPK inhibition were seen for all genes tested. Statistical analysis was not possible due to the small number of experiments.

## **DISCUSSION**

In this study, the effect of p38 MAPK inhibition on the acute phase response was investigated in hepatoma cell lines. We found significant inhibition of CRP and fibrinogen production at 1  $\mu\text{M}$  RWJ 67657, which is considered a pharmacologically relevant concentration.

The importance of the cytokines IL-1 $\beta$  and TNF- $\alpha$  in inflammation has been established, particularly by the success of cytokine blockade in the treatment of RA. Also the use of anti-IL-6 antibody intended to block the JAK/STAT pathway seems promising<sup>22</sup>. The interest in the regulation of cytokine production by signal transduction pathways and transcription factors has led to new therapeutic targets<sup>23</sup>. For RA several p38 MAPK inhibitors have been designed, which have been tested pre-clinically and in some cases even in clinical studies<sup>13;16</sup>. In clinical trials, responses are measured by using the American College of Rheumatology (ACR) criteria and several laboratory markers, including acute phase proteins. It is important to know whether the levels of acute phase proteins are not directly blocked by therapeutic agents.

Stimulation of hepatoma cells with IL-6 induced a weak p38 MAPK phosphorylation after 1 hour, in contrast to the rapid and strong phosphorylation by IL-1 $\beta$  and TNF- $\alpha$  stimulation. These cytokines could also induce a weak STAT3 phosphorylation in hepatoma cells, but only at a late time point (24 hours).

In contrast to others<sup>24;25</sup> it was not possible for us to detect CRP production or mRNA expression by HepG2 and HuH7 cells making these cells less valuable as model to study acute phase reactions. Induction of CRP production and mRNA expression however was found in Hep3B and PLC/PRF/5 cells especially after combined IL-6 and IL-1 $\beta$  stimulation (in PLC/PRF/5 also after IL-6 and TNF- $\alpha$  stimulation). Pre-treatment with the p38 MAPK inhibitor at 1  $\mu\text{M}$  did significantly reduce CRP production. The study by Parasrampur<sup>26</sup> demonstrated that after a single oral dose of RWJ 67657 ranging from 0.25 to 30 mg/kg a plasma concentration of 0.01 to 6  $\mu\text{M}$  of the p38 MAPK inhibitor could be reached in humans, so 1  $\mu\text{M}$  is a pharmacologically relevant concentration. Recent studies investigating prognostic factors in RA<sup>27</sup>, but

also work from our group <sup>4</sup>, have demonstrated that levels of CRP especially seem to be predictive for joint damage.

All 4 cell lines showed high production of SAA, again especially after combined IL-6 and IL-1 $\beta$  stimulation, and inhibition by RWJ 67657 was seen only at 10  $\mu$ M. SAA induction was also seen in PLC/PRF/5 after IL-6 and TNF- $\alpha$  stimulation and inhibition of SAA production at 1  $\mu$ M RWJ 67657 was seen in HuH7 cells after IL-1 $\beta$  stimulation. Strong induction of SAA-1 mRNA was demonstrated in PLC/PRF/5 and Hep3B after combined IL-6 and IL-1 $\beta$  stimulation. Our results confirmed the data reported by Hagihara *et al* <sup>28</sup>, concerning the critical role of IL-6 in the synergistic induction of SAA gene expression in hepatoma lines. Moreover they found that anti-IL-6R Mab almost completely inhibited the synergistic increase of SAA-1 after triple cytokine stimulation, while IL-1ra and anti-TNF- $\alpha$  Mab only had moderate effects. In our study, the use of a p38 MAPK inhibitor had no effect on SAA production and expression, in contrast to the effects on CRP.

Fibrinogen synthesis is mainly mediated by IL-6, and this was shown in our studies in the 4 hepatoma cell lines. Combined stimulation induces lower fibrinogen production, due to cross-talk between IL-6 and IL-1 $\beta$  signalling pathways leading to IL-1 $\beta$  dependent down-regulation of STAT1 phosphorylation <sup>29</sup>. p38 MAPK treatment additionally reduced fibrinogen production independent of which stimulus was used. Fibrinogen plays an important role in the coagulation cascade, but is also an important acute phase protein, because the often-used ESR largely depends on the fibrinogen concentration. The fact that indeed fibrinogen production is reduced by p38 MAPK treatment could have important implications for the use of ESR as marker of disease activity.

Finally, complement production was reduced in all cell lines at 10  $\mu$ M RWJ 67657, but no effect on mRNA expression was seen. Albumin mRNA expression was reduced after stimulation with cytokines as expected, and no effect of p38 MAPK treatment was demonstrated.

In human liver slices control incubations demonstrated a spontaneous release of acute phase proteins. This has been shown before <sup>19</sup> and may be related to the source of the human liver. The human liver slices are prepared from livers from brain-dead donors, and it is known that e.g. CRP is up regulated after brain-death, due to high levels of IL-6 <sup>30</sup>. We have shown increased levels of IL-6 in unstimulated slice culture medium after 24 hours, which were further increased after LPS stimulation <sup>31</sup>. However, incubation of the human liver slices with IL-6 and IL-1 $\beta$  still led to an increase of the release of acute phase proteins. These results indicate there is induction of the acute phase proteins release possible as was seen in previously performed studies <sup>19</sup>. However, in contrast to the results of the hepatoma cell lines, pre-incubation with RWJ 67657 did not lead to a significant reduction in acute phase proteins in human liver slices, although a tendency was seen in the case of fibrinogen. In humans a plasma concentration of 6  $\mu$ M RWJ 67657 can be achieved after a single oral dose. In our study with liver slices we only used a concentration of 1  $\mu$ M, which could be too low to inhibit production of for instance CRP.

CRP and SAA are the major acute phase reactants in rheumatoid arthritis, but they have a different function and are differentially regulated <sup>2</sup>, although both CRP and SAA are potently induced by combined IL-6 and IL-1 $\beta$  stimulation. The fact that CRP

production is inhibited at the pharmacological relevant concentration of 1  $\mu$ M RWJ 67657, while SAA production is not, might be explained by differences in the acute phase responsive elements in the promoter regions of the two APP<sup>32</sup>. Cunnane *et al*<sup>33</sup> demonstrated that SAA is the best marker for the assessment of inflammatory joint disease, while Rau *et al* demonstrated that in acute pancreatitis SAA had a wider dynamic range, but measurement of CRP provided an earlier differentiation between patients<sup>34</sup>. The results presented in this study may implicate that SAA measurement should be included in clinical trials when p38 MAPK inhibitors are used.

## CONCLUSIONS

In human hepatoma cell lines a significant inhibition of CRP and fibrinogen production and mRNA expression was found after treatment with a p38 MAPK specific inhibitor at pharmacologically relevant concentrations. SAA production however was not reduced in these cell lines. In human liver slices, which mimics an *in vivo* model, fibrinogen production was also reduced. As p38 MAPK inhibitors are now in phase II clinical trials, we encourage to include the use of SAA as marker for disease activity, because this acute phase protein does not seem to be affected directly by p38 MAPK treatment.

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